# CAVEOLIN-1 IS NOT REQUIRED FOR THE ACCUMULATION OF POLYCHLORINATED BIPHENYLS IN ADIPOCYTES IN VITRO

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**Introduction**

Caveolae are 25-150 nm small invaginations in the plasma membrane that are abundant in endothelial cells, fibroblasts, skeletal muscle cells and particularly in adipocytes[1]. These microdomains of the cell membrane are lipid rafts, enriched in cholesterol and sphingolipids and are stabilized by their major coat constituent, caveolins. Caveolins are intramembrane proteins made of 3 members, caveolin-1, -2 and -3. Caveolin-1 is especially abundant in the adipose tissue. In cultured adipocytes, caveolar endocytosis has been shown to be targeted to lipid droplets upon addition of cholesterol and exogenous fatty acids[2,3], suggesting a possible role for lipophilic molecules in the stimulation of this pathway. More recently, caveolae formation was shown to be induced by a polychlorinated biphenyl (PCB) congener, PCB-77, in endothelial cells, suggesting that caveolae could be a critical uptake site for such toxic, lipophilic pollutants[4]. PCBs are persistent environmental pollutants that massively accumulate in the adipose tissue of contaminated organisms due to their highly lipophilic properties. These organic contaminants have shown numerous harmful effects on animal and human health[5,6]. Although the adipose tissue constitutes the major intern reservoir of PCBs, there is little precise information about the mechanisms involved in the uptake of these molecules by adipocytes. Caveolin-1 being highly expressed in adipocytes, we investigated the possible role of caveolar endocytosis in the uptake of PCBs in cultured adipocytes. First, we followed the accumulation of 2,4,4'-trichlorobiphenyl (PCB-28), 2,3',4,4',5-pentachlorobiphenyl (PCB-118) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) in differentiated adipocytes derived from mouse embryonic fibroblasts (MEFs) obtained from wild-type (WT) and caveolin-1 KO (cav-1 KO) mice. Second, we compared the accumulation of the same cocktail of PCBs in differentiated 3T3-L1adipocytes overexpressing caveolin-1 and controls. Third, we contaminated mature adipocytes derived from WT and cav-1 KO mice and then isolated the lipid droplets in order to localize the cellular compartments in which the PCBs accumulated.

**Materials and methods**

*Cell culture*.

- Cav-1 KO and WT MEFs were prepared as described elsewhere[2,7] from 13.5 p.c embryos coming from caveolin-1 KO and WT mice respectively. Adipocyte differentiation was then induced during 48h by adding isobutylmethylxanthine (IBMX, 500µM), dexamethasone (Dex, 1,25 µM), insulin (870 nM), penicillin (100U/ml) and streptomycin (0.1 mg/ml) to the medium. For the rest of the differentiation process, IBMX and Dex were removed and rosiglitazone (0,5 µM) was added to the differentiation inducer cocktail every 48h during 5-6 days until experiments.
- 3T3-L1 cells (kind gift of Dr J. Pairault, Paris, France) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) until 2 days post-confluence (day 0). Adipocyte differentiation was then induced as described for MEFs except for IBMX (500 µM) and insulin (870 nM) initial concentrations. After 48h of incubation, the medium was removed and the cells were incubated for another 48h in the medium just described but without IBMX and Dex and with lower insulin concentration (100 nM). For the rest of the differentiation process, insulin was also suppressed from the medium which was then replaced every 48h until 10-12 days post-confluence. To achieve overexpression of caveolin-1, differentiated adipocytes were transfected for 48h at 37°C-10% $CO_2$ with an adenovirus encoding either for caveolin-1 (cav-1 cells) or for the Green Fluorescent Protein (GFP). The latter were used as transfection controls (null-GFP cells).

- Once differentiated into adipocytes, 3T3-L1 cav-1/null-GFP and MEFs WT/cav-1 KO cells were incubated for 30', 90', 4h and 8h at 37°C – 10% $CO_2$ with a cocktail of 3 PCB congeners, PCB-28, PCB-118 and PCB-153. PCBs were added to the cell medium as an ethanolic solution in order to obtain a final concentration of 500 nM for each congener. Control cells received the ethanol vehicle alone that did not represent more than 0,5% vol.

*Isolation of lipid droplets*.
Mature adipocytes were isolated from epididymal adipose tissue obtained from caveolin-1 KO and WT mice, after an hour-long digestion at 37°C in a collagenase solution (0,15 U/ml). Once isolated, the cells were incubated for 2h at 37°C with the PCB cocktail described above. After incubation, lipid droplets were isolated as described in detail elsewhere[2].

*Biochemical and chemical analyses*.
All cell conditions were used for western-blotting in order to verify the absence of caveolin-1 in KO conditions and to control the overexpression of the protein in the transfected 3T3-L1 adipocytes. PCBs were analyzed by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a $^{63}$Ni ECD detector. Details of the procedure are described elsewhere[8]. Total cellular glycerolipids were measured, after the chemical saponification of the cell lysates in a 0.1M KOH-MeOH solution for 1h at 70 °C, via the quantification of released glycerol using a commercially available kit (Free glycerol FS, DiaSys). Phospholipids (PL) were quantified by gas chromatography and enabled to calculate the amounts of triglycerides (TG) present in the cells. Protein contents were determined using the Bicinchoninic Acid (BCA) protein assay kit (Sigma-Aldrich).

**Results and discussion**
Results obtained for PCB-153 are presented here. The two other congeners tested in the present study (PCB-28 and PCB-118) have shown the same results.

- *Validation of all cell models for absence/presence/overexpression of caveolin-1*.
As shown by western-blotting in Fig. 1, caveolin-1 was undetected in cav-1 KO MEFs and mature adipocytes from cav-1 KO mice whereas it was clearly expressed in both WT cultured MEFs and isolated adipocytes (Fig. 1- MEFs – Epi). In order to verify the extent of the overexpression of caveolin-1 in the transfected cells, we controlled its levels in cav-1 and null-GFP 3T3-L1 differentiated adipocytes (Fig.1-3T3-L1). Results confirmed that the cav-1 3T3-L1 cells expressed significantly higher levels of caveolin-1 than the null-GFP condition.

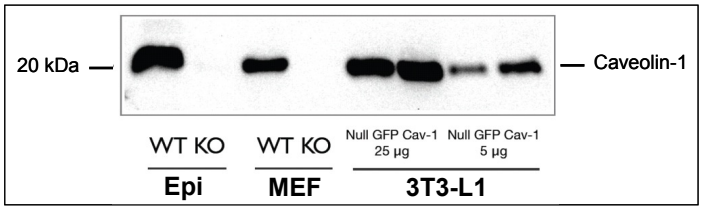


Figure 1: Western Blot of isolated adipocytes from epididymal fat pads coming from cav-1 KO and WT mice (Epi), cav-1 KO and WT MEFs differentiated into adipocytes (MEF) and 3T3-L1 adipocytes overexpressing caveolin-1 as compared to control cells (3T3-L1).

- *Accumulation of PCB-153 in Cav-1 KO/WT MEFS differentiated into adipocytes*.
When accumulated amounts of PCBs are expressed per unit of proteins (Fig. 2A), we observed a significant difference between both cell conditions, with WT MEFs storing twice more PCB-153 throughout the 8 hour-long incubation period than the cav-1 KO adipocytes. However, caveolin-1 does not seem essential for PCB-153 accumulation as up to 40% of the initially added amounts of PCB-153 in the medium of cav-1 KO MEFs did accumulate in these cells. Moreover, when all these results were expressed per unit of TG (Fig. 2B), the differences observed in Fig.2A disappeared and the accumulated amounts of contaminants in both cell types became similar. WT MEFs containing more TG than cav-1 KO MEFs (results not shown), the higher amounts of PCB-153 in WT MEFs could rather be explained by the higher levels of intracellular lipids than by the presence of caveolin-1 in these cells.

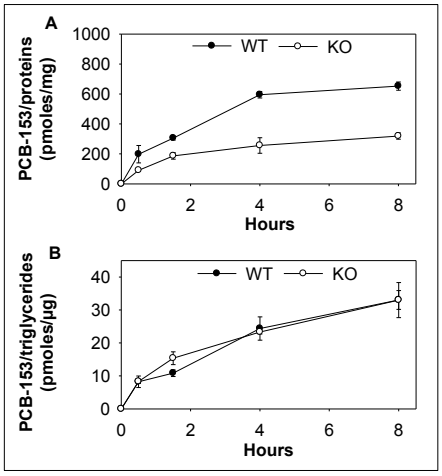


Figure 2: Accumulation rate of PCB-153 added at a concentration of 500 nM in the medium of caveolin-1 KO and WT MEFs differentiated into adipocytes. Results are expressed per unit of proteins (A) and triglycerides (B).

- *Accumulation of PCB-153 in Cav-1/Null GFP 3T3-L1 differentiated adipocytes*.
When the amounts of accumulated PCB-153 were expressed per unit of proteins (Fig. 3A), we observed no difference between both cell types. The higher levels of caveolin-1 in the cav-1 overexpressing cells did not lead to a higher accumulation rate of PCB-153. To the contrary of cav-1 KO and WT MEFs, TG levels between these cell conditions were not different. As a consequence, we observed no significant difference of accumulation between both conditions throughout the incubation period, when the amounts of PCB-153 were expressed per unit of TG (Fig. 3B). This result is thus in accordance with the previous observation in MEFs, indicating that the higher accumulation rates of PCBs were dictated by the levels of cellular lipids rather than by caveolin-1.

- *Intracellular localization of PCB-153 in isolated mature adipocytes from cav-1 KO and WT mice*.
Caveolin-1 has been proposed to be involved in cellular trafficking from the plasmic membrane to the lipid droplets upon fatty acid or cholesterol exposure of culture cells[3,4] These observations suggest a possible implication of caveolin-1 in the incorporation of lipophilic molecules such as PCBs into the fat droplets. However, results obtained from isolated WT and cav-1 KO adipocytes showed that, in both cases, 99% of the accumulated amounts of PCB-153 were associated with the lipid droplet fraction, indicating that caveolin-1 was not required for the transfer of PCBs into this intracellular compartment.

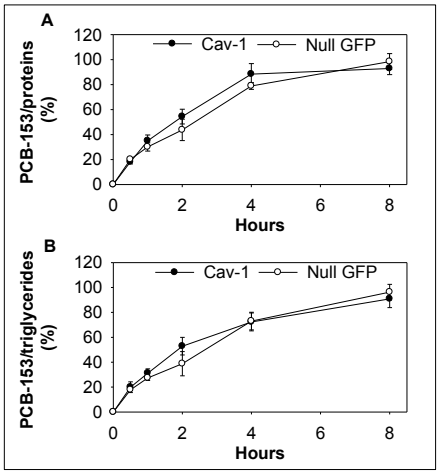


Figure 3: Accumulation rate of PCB-153 added at a concentration of 500 nM in the medium of differentiated 3T3-L1 adipocytes overexpressing caveolin-1 (cav-1) and controls (null GFP). Results are expressed as the percentage of PCB-153 accumulated in cells per unit of proteins (A) and per unit of triglycerides (B).

Taken together, these results clearly indicate that caveolin-1 was not necessary for the entrance of PCBs in cultured and mature adipocytes and was also not required for the intracellular trafficking of these pollutants from the plasmic membrane to the lipid droplet compartments.

**Acknowledgements**

We are very grateful to F. Lasnier and X. Le Liepvre from the "Centre de Recherches Biomédicales des Cordeliers", Paris, France, for technical assistance in diverse assays and for the cell cultures. We would also like to acknowledge M. Louvet from the "Laboratoire d'Ecologie animale et d'Ecotoxicologie", Université de Liège, Belgium, for her help in PCB analyses. We also thank A. Joly from the "Laboratoire de biochimie cellulaire, nutritionnelle et toxicologique", Institut des Sciences de la Vie, Université catholique de Louvain, Belgium, for her help in the fatty acid analyses.

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